CHOLESTEROL REFERENCE METHOD LABORATORY NETWORK

Sample Stability Protocol

Background

The Cholesterol Reference Method Laboratory Network (CRMLN) developed certification protocols for total cholesterol, HDL cholesterol, and LDL cholesterol. All of these protocols state:

> The CRMLN strongly recommends that manufacturers set aside and store (*at -70°C or lower*) additional aliquots of each fresh sample (volume consistent with analytical system requirements). These samples can be used for reanalysis if changes in calibration are required to meet certification criteria. When new lots of calibrators, materials, or reagents are prepared, these frozen samples can provide an important link to the accuracy base during overlap analyses if a frozen versus fresh comparison has been performed.

This protocol is designed as a guideline for manufacturers to use in comparing results from frozen samples versus fresh samples. The CRMLN will not evaluate the data collected from sample stability studies. This protocol is provided as a guideline for manufacturers who would like to save additional aliquots of samples used in CRMLN certification protocols. It is in the best interest of the users of this protocol to follow it carefully so that undue errors are not generated in future certification attempts.

Quality Control

The user of this protocol will need to know the coefficient of variation (CV) of the analytical method to be able to determine a sample size for the study. This information is obtained from the quality control (QC) data.

In addition to providing a CV for determining the sample size for the study, the QC is the key to having a valid stability study. The manufacturer must have a stable QC system in place before beginning the stability study. A tacit assumption in QC is that the material is stable over time. The best material for QC will be one that has been shown to be stable for the duration of the study. This establishes the method's stability base. The material is also expected to be stable over the time frame being studied so that the method's stability can be verified. Before beginning, the manufacturer should have collected QC data that covers the same length of time planned for the study. It is advisable that the QC material be measured in additional runs during the course of time that the test samples are frozen to insure that the method remains stable during the study. This will give the study the statistical power to demonstrate that any changes observed are due to changes in the fresh-frozen materials and not due to changes in the analytical method. It is also advisable to have data from several levels of QC materials to determine if the variance is uniform over the analytical range being studied.

The QC characterization data should, ideally, be collected using a single lot of calibrator and a single lot of reagent. Minimally, this same lot of calibrator must also be used for the stability study.

Statistical Approach

The number of samples required to adequately detect a difference depends on the CV of the analytical method. For 80% power to detect a 1% difference, the following numbers of samples are needed for various analytical CVs:

CV	# samples	CV	# samples	CV	# samples
1.0	18	2.0	65	3.0	144
1.1	22	2.1	72	3.1	153
1.2	25	2.2	78	3.2	163
1.3	29	2.3	86	3.3	173
1.4	33	2.4	93	3.4	184
1.5	38	2.5	101	3.5	195
1.6	43	2.6	109	3.6	206
1.7	48	2.7	117	3.7	217
1.8	53	2.8	126	3.8	229
1.9	59	2.9	134	3.9	241

The t-test will be used to analyze the data. The t-test uses an estimate of variance (standard deviation, SD) in the calculation, not CV. Use of the t-test assumes that the variance is uniform across the analytical range. If the variance is not uniform across the analytical range (i.e. if the SD has concentration dependence), then the range must be divided into concentration regions with uniform variance. Each concentration region will require the appropriate number of samples from the table above, depending on the CV of the individual region.

This protocol is written for a method with uniform variance and an analytical CV of 1%. If the method has a larger CV, then both the number of samples per day and the number of days when samples are collected will necessarily need to be increased.

Protocol

The protocol is written for serum. However, it can be easily adapted for plasma if that is the matrix used with the analytical system being evaluated. Three time frames are included in the protocol – frozen for 7, 30, and 60 days. However, the protocol can also be adapted to include

additional or different intervals. If additional durations are to be evaluated, a larger volume of each individual sample will be needed. Conversely, if fewer time frames are to be evaluated, a smaller volume of each individual sample will be needed.

It is critical that the entire sample stability study be conducted with a single lot of reagent and a single lot of calibrator. All of the samples studied and all of the QC materials must be analyzed using the same lots of reagent and calibrator. It is also critical that the QC characterization runs and the sample stability study be conducted with, minimally, the same lot of calibrator. Ideally, the same lot of reagent should also be used for the QC characterization runs and the sample stability study, but this may be more difficult to organize.

Samples will be collected in multiple cycles, starting on separate days, to simplify the collection process.

Follow the collection guidelines described in the "Manufacturer's Specimen Collection" section in the individual protocol of interest (e.g. total cholesterol, HDL cholesterol, LDL cholesterol, or triglyceride). See the Appendix of this protocol for the concentration distribution for each analyte.

On the first day, collect blood from a subset of the total number of donors. For each individual donor, harvest the serum and pool the serum if more than one tube was collected. Divide the serum into 4 aliquots (or more if additional time frames are to be investigated). Freeze 3 aliquots at -70EC. Within 4 hours of collection, analyze the fourth aliquot in duplicate by the routine clinical method. At 7, 30, and 60 days, remove an aliquot from the freezer and analyze it in duplicate by the routine clinical method.

On additional days, collect blood from another group of donors. Repeat the protocol as on the first day.

Be sure to keep the samples from the multiple collection cycles separate to avoid confusion.

During the time frame that the samples are frozen, run the method with the QC materials every other day. Use the same lot of reagents and calibrator during this time period.

A sample of a table that can be used to record data is included with this protocol.

After all samples have been analyzed, combine the data for the 7-days frozen from each collection cycle. Evaluate the results by the t-test, comparing the 7-days frozen group to the fresh group. Likewise, combine the data for the 30-days frozen from each collection cycle and evaluate it using the t-test. Finally, combine the data for the 60-days frozen from each collection cycle and evaluate it using the t-test.

t-test

Perform the t-test for each interval separately (e.g. 7-days frozen v. fresh). Calculate the average of the duplicate measurements performed for each sample and time.

$$average_{fresh_i} = \left(R1_{0_i} + R2_{0_i}\right)/2$$

where $R1_{0_i}$ and $R2_{0_i}$ are the replicates for the fresh sample.

$$average_{7_i} = \left(R1_{7_i} + R2_{7_i}\right)/2$$

where $R1_{7_i}$ and $R2_{7_i}$ are the replicates for the 7-days frozen sample.

For each sample, calculate the difference between the frozen aliquot and the fresh aliquot from the averages of the duplicates.

$$difference_i = average_{7_i} - average_{fresh_i}$$

For each time frame (i.e. 7-, 30-, and 60-days frozen), calculate the average difference for all of the samples in the concentration range.

$$avgdiff_{7days} = \frac{\sum_{i=1}^{n} difference_i}{n}$$

where n is the number of samples in the concentration range.

Calculate the SD_{diff} for the paired differences.

$$SD_{diff} = \sqrt{\frac{\sum_{i=1}^{n}\left(difference_i\right)^2 - n(avgdiff_{7days})^2}{n-1}}$$

Use the paired t-statistic formula as follows:

$$t = \frac{\left(avgdiff_{7days}\right)\sqrt{n}}{SD_{diff}}$$

Use a table of "critical values for t" for a 2-tailed **"** = 0.05 and degrees-of-freedom = n – 1. If the value calculated for t is greater than the critical value, then there is a significant difference between the frozen and fresh samples.

Table 1: Sample Stability Data

Start Date	Sample ID	Fresh Results		Frozen 7 Days		Frozen 30 Days		Frozen 60 Days	
		$Rep1_0$	$Rep2_0$	$Rep1_7$	$Rep2_7$	$Rep1_{30}$	$Rep2_{30}$	$Rep1_{60}$	$Rep2_{60}$

Table 2: Quality Control Limits

Record previously determined QC limits in this table.

Material	Mean	99% LCL	95% LCL	95% UCL	99% UCL	95% RL	99% RL

LCL: lower control limit
UCL: upper control limit
RL: Range limit

Table 3: Quality Control Results
Material 1

Material	Date	Result #1	Result #2	Mean	Range	Mean in Control?	Range in Control?

Table 4: Quality Control Results
Material 2

Material	Date	Result #1	Result #2	Mean	Range	Mean in Control?	Range in Control?

Appendix: Concentration distribution

These concentration distributions are based on using 18 samples for the comparison which assumes an analytical CV of 1%.

Total Cholesterol
Distribute the samples using the following guidelines:

- 20% samples from 120 to 180 mg/dL (3.10 to 4.67 mmol/L)
- 30% samples from 181 to 220 mg/dL (4.68 to 5.71 mmol/L)
- 30% samples from 221 to 260 mg/dL (5.72 to 6.74 mmol/L)
- 20% samples from 261 to 400 mg/dL (6.75 to 10.34 mmol/L).

HDL Cholesterol
Approximately 60% of the samples should be divided equally among each of the following ranges. The remaining samples can fall into any of the five ranges; care should be taken to distribute the remaining samples over the concentration range.

Range	
20-29 mg/dL	(0.52-0.77 mmol/L)
30-39 mg/dL	(0.78-1.03 mmol/L)
40-49 mg/dL	(1.04-1.29 mmol/L)
50-59 mg/dL	(1.30-1.55 mmol/L)
60-69 mg/dL	(1.56-1.81 mmol/L)

LDL Cholesterol
Distribute the samples using the following guidelines:

- 20% of samples < 100 mg/dL (2.59 mmol/L)
- 30% of samples from 100 to 130 mg/dL (2.59 to 3.36 mmol/L)
- 30% of samples from 131 to 160 mg/dL (3.37 to 4.14 mmol/L)
- 20% of samples from 161 to 400 mg/dL (4.15 to 10.35 mmol/L)

Triglyceride
Distribute the samples using the following guidelines:

- 10% samples < 75 mg/dL (< 0.85 mmol/L)
- 25% samples from 75 to 124 mg/dL (0.85 to 1.40 mmol/L)
- 30% samples from 125 to 199 mg/dL (1.41 to 2.25 mmol/L)
- 25% samples from 200 to 299 mg/dL (2.26 to 3.38 mmol/L)
- 10% samples from 300 to 400 mg/dL (3.39 to 4.52 mmol/L)